LAWRENCE LIVERMORE NATIONAL LABORATORY

LLNL-JRNL-443171

Prospective Analysis of DNA Damage and Repair Markers of Lung Cancer Risk from the Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening Trial

A. J. Sigurdson, I. M. Jones, Q. Wei, X. Wu, M. R. Spitz, D. Stram, M. D. Gross, W. Huang, L. Wang, J. Gu, C. B. Thomas, D. Reding, R. B. Hayes, N. E. Caporaso

July 19, 2010

Carcinogenisis

PROSPECTIVE ANALYSIS OF DNA DAMAGE AND REPAIR MARKERS OF LUNG CANCER RISK FROM THE PROSTATE, LUNG, COLORECTAL, AND OVARIAN (PLCO) CANCER SCREENING TRIAL

Alice J. Sigurdson[1]*, Irene M. Jones[2], Qingyi Wei[3] Xifeng Wu[3], Margaret R. Spitz[3], Douglas Stram[1], Myron D. Gross[4], Wen-Yi Huang[5], Li-E Wang[3], Jian Gu[3], Cynthia B. Thomas[2], Douglas Reding[6], Richard B. Hayes[5,7], Neil E. Caporaso[8]

[1]Radiation Epidemiology Branch, Division of Cancer Epidemiology and Genetics, National Cancer Institute, NIH, DHHS, Bethesda, MD
[2] Lawrence Livermore National Laboratory, Livermore, CA
[3]Department of Epidemiology, The University of Texas M. D. Anderson Cancer Center, Houston, TX
[4]University of Minnesota, Minneapolis, MN
[5]Occupational and Environmental Epidemiology Branch, Division of Cancer Epidemiology and Genetics, National Cancer Institute, NIH, DHHS, Bethesda, MD
[6]Department of Oncology and Hematology, Marshfield Clinic, Marshfield, WI
7 Division of Epidemiology, Department of Environmental Medicine, New York University Langone Medical Center, New York, NY
[8]Genetic Epidemiology Branch, Division of Cancer Epidemiology and Genetics, National Cancer Institute, NIH, DHHS, Bethesda, MD

*Correspondence and reprint requests to: Alice J. Sigurdson, Radiation Epidemiology Branch, Division of Cancer Epidemiology and Genetics, National Cancer Institute, NIH, DHHS, 6120 Executive Boulevard, EPS 7060, MSC 7238, Bethesda, MD, USA, 20892-7238

TEL: 301.594.7911

FAX: 301.402.0207

EMAIL: sigurdsa@mail.nih.gov

Key Words: Mutagen sensitivity, DNA repair, Comet assay, lung cancer, genetic variation, pre-diagnostic samples, risk factors, prospective study

Abstract = 249 words
Text = 2946 words

Abstract

Mutagen challenge and DNA repair assays have been used in case-control studies for nearly three decades to assess human cancer risk. The findings still engender controversy because blood was drawn after cancer diagnosis so the results may be biased; a type called “reverse causation”. We therefore used Epstein Barr virus-transformed lymphoblastoid cell lines established from prospectively collected peripheral blood samples to evaluate lung cancer risk in relation to three DNA repair assays: alkaline Comet assay, DNA repair capacity with the mutagen benzo(a)pyrene diol epoxide, and the bleomycin mutagen sensitivity assay. Cases (n=117) were diagnosed with lung cancer between 0.3 and 6 years after blood collection and controls (n=117) were frequency matched on age at blood collection, gender, and smoking history; all races were included. Case and control status was unknown to laboratory investigators. In unconditional logistic regression analyses, statistically significant increased lung cancer odds ratios ($OR_{adjusted}$) were observed for bleomycin mutagen sensitivity as quartiles of chromatid breaks/cell (relative to the lowest quartile, OR=1.2, 95% confidence interval (CI): 0.5-2.5, OR=1.4, 95% CI: 0.7-3.1, OR=2.1, 95% CI: 1.0-4.4), respectively, $p_{trend} = 0.04$). The magnitude of the association between the bleomycin assay and lung cancer risk was modest compared to those reported in previous lung cancer studies but was strengthened when we included only incident cases diagnosed more than a year after blood collection ($p_{trend} = 0.02$), supporting the notion the assay may be a measure of cancer susceptibility. The Comet and DNA repair capacity assays were unrelated to lung cancer risk.

Introduction

Mutagen challenge assays were introduced in the early 1980's (1-4) and since then several hundred case-control study results have reported various measures of DNA damage or functional tests of DNA repair capacity were associated with two- to 10-fold increased cancer risk at several sites (reviews in 5-10). All of these case-control studies shared the design limitation that the assays are unable to disentangle the host's response to cancer and the postulated underlying genetic susceptibility. This limitation has been termed "reverse causation bias". The reverse causation bias problem has been thoughtfully discussed in several reviews and editorials (5, 8-9, 11-13), with the suggested solution to conduct prospective or nested case-control studies with stored pre-diagnostic samples. A prospective study with assay determination on fresh (unfrozen) peripheral blood samples for a large cohort of subjects followed for cancer outcomes is prohibitively expensive because the assays are labor intensive. Nested studies using cryopreserved lymphocytes or blood may be promising (14-15) but laboratory cell culturing and other technical challenges of using thawed samples remain problematic (16). To our knowledge, one small mutagen sensitivity study followed cancer-free individuals with Barrett's esophagus, finding a non-significantly 1.6-fold increased risk of esophageal carcinoma (17). Other supporting evidence that mutagen challenge assays measure inherent and tissue-specific cancer susceptibility include heritability and twin studies (reviewed in 8), reports of similar findings of peripheral blood cells and target organ tissue (reviews in 7, 9-10), stability of the assay over time (reviews in 9) and in pre- and post-diagnosis samples (18), and case-only analyses for second tumor and recurrence risk (reviewed in 8; 19). Despite this indirect evidence, prospectively designed studies are the only means to definitively determine whether DNA damage or mutagen challenge assays are an unbiased measure of underlying cancer predisposition.

We generated Epstein Barr virus (EBV)-transformed lymphoblastoid cell lines (LCLs) from peripheral blood samples collected before diagnosis to analyze lung cancer risk using three separate assays that are considered to assess base excision (20); nucleotide excision (21) and double strand break repair pathways (2), respectively: the alkaline Comet Assay, the host cell reactivation assay with the activated mutagen benzo(a)pyrene diol epoxide (BPDE), and the bleomycin mutagen sensitivity assay. Cryopreserved whole blood samples have been collected from more than 50,000 participants in the Prostate, Lung, Colorectal and Ovarian Cancer (PLCO) Screening Trial and was the population base for the 117 incident lung cancers and 117 controls without lung cancer studied here.

Materials and methods

Study population and blood collection

PLCO study design and biospecimen collection methods have been published previously (22-24). In brief, the PLCO study is a randomized screening trial with the objective to measure the effect of periodic diagnostic screening on prostate, lung, colorectal, and ovarian cancer incidence and mortality. The subjects in the trial are 154,938 men and women who were aged 55 to 74 years and were free of the studied cancers at time of entry into the study. In one arm, individuals were followed as they underwent usual care, while the other arm had additional screening tests for the cancers of interest as well as usual care. Blood samples were collected from subjects in the screening arm at prescribed intervals over the course of the trial including cryopreserved whole blood samples used in the present study. Maintenance of lymphocyte viability and successful EBV transformation, up to several years after collection, have been previously reported (23).

Cancer case and control selection

Cases were individuals with lung cancer diagnosed between three months and six years after whole blood collection and were not restricted by lung cancer histology. Controls without lung cancer were frequency matched to cases by gender, age at blood collection, calendar year of blood collection, and smoking history (never, quit 10+ years ago and cigarettes/day ≤ 1 pack, quit 10+ years ago and cigarettes/day ≥ 1 pack, current smoker or quit < 10 years ago and cigarettes/day ≤ 1 pack, or current smoker or quit < 10 years ago and cigarettes/day > 1 pack). All participants gave informed consent. This study has been approved annually by the human subjects review boards of the National Cancer Institute and the individual institutions contributing to the PLCO trial. Studies conducted at Lawrence Livermore National Laboratory and The University of Texas M. D. Anderson Cancer Center were approved by their respective Institutional Review Boards.

Samples

A lymphoblastoid cell line was prepared by EBV transformation of peripheral blood lymphocytes obtained from each subject. All stored samples were successfully transformed and each cell line was cryopreserved. Study samples were shipped in dry ice shippers to the study laboratories and tracked by a unique ID code. Laboratory investigators had no knowledge of case or control status, age, gender, ethnicity, smoking history, or descriptive information for any of the samples. Each cell line sample was thawed and cultured in RPMI 1640 supplemented with 15% serum (Fetal Clone III, HyClone, Logan, Utah) and 2mM glutamine prior to analysis. The period of culture prior to analysis varied among cell lines, from a few days to weeks, depending on the growth rate of the cell line and the proportion of viable cells measured by trypan blue dye exclusion. In general, approximately 70% of the cell lines grew within one week. For quality control (QC) assessment, four replicate samples of two individuals and duplicates from eight individuals were included in each shipment. Laboratory personnel were blinded to the identity of these replicate samples.

Measurement of DNA damage.

Comet Assay

The alkaline single cell gel electrophoresis (Comet) assay quantitatively measures the amount of DNA single strand breaks (SSBs) in individual cells. The assay reflects endogenous DNA damage and therefore high values are thought to correspond to an increased amount of cellular DNA strand breakage and/or alkali-labile sites. For the present study, the Comet assay (20) was performed with slight modifications as described previously (25). Briefly, cells were suspended in 0.5% low melting point agarose and spread on each of two slides and treated in the dark at 4°C with lysis buffer overnight then rinsed. Slides were then placed in the electrophoresis unit and covered with a fresh solution of 300 mM NaOH, 1 mM EDTA, final pH >13.0, for 60 min. The slides were electrophoresed at 0.92V/cm (28 volts/30.5cm) with current adjusted to 300 mamps for 25 min. Images of 50 cells on each of 2 slides were captured and comet parameters determined using Komet4.0©: Image Analysis and Data Capture software (Kinetic Imaging, Ltd., Merseyside, England). Four comet parameters were analyzed: "Tail DNA" is the percent of DNA (fluorescence) in the tail. "Tail length" is the length of the tail in μm, measured from the leading edge of the head; Comet Distributed Moment (CDM), also referred to as comet moment, is the moment of fluorescence of the whole comet and does not distinguish head and tail; Olive Tail Moment (OTM) is the percentage of DNA in the tail (tail DNA) times the distance between the means of the tail and head fluorescence distributions. Both CDM and OTM are expressed in arbitrary units. Higher values of the comet parameters are hypothesized to indicate increased cancer susceptibility.

Host Cell Reactivation Assay

The Host Cell Reactivation assay can be used to measure cellular DNA repair capacity (DRC) based on the principle that if a reporter gene is damaged before transfection, its expression in a cell is dependent on the ability of the host cell to repair the damage. The repair capacity of LCLs is assumed to reflect the repair capacity of the donor, because in the DNA repair deficiency syndrome xeroderma pigmentosum, low DRC is detected in many tissues including lymphocytes and their derived cell lines (26). To measure the cells' ability to remove tobacco-carcinogen (BPDE)-induced DNA damage in a reporter gene encoding luciferase (LUC) in the plasmid pCMV*luc*, LCL cells from the subjects were transfected with untreated and 60 μM BPDE treated plasmids in parallel (21; 26-27). The cultures were then incubated for 40 h after transfection. LUC activity was measured in arbitrary light-intensity units and was recorded for the cells with undamaged plasmids (control reading) and BPDE-damaged (repair reading) plasmids. The DRC (in percent) is a ratio of the light intensity in BPDE-damaged plasmids to that of the undamaged plasmids X 100. Higher values of DRC are hypothesized to indicate decreased cancer susceptibility.

Bleomycin mutagen sensitivity assay

The bleomycin mutagen sensitivity assay was conceived and developed by T. C. Hsu in the early 1980s (1-2). The assay was designed to identify and measure indicators of genetic susceptibility based on quantifying the extent of chromosome breakage induced by the radiomimetic agent, bleomycin. Cultured LCL cells from subjects were treated with bleomycin (final concentration, 0.03 U/mL) (Blenoxane: Nippon Kayaku Co., Ltd.). At 71 hours, 0.04 μg/mL colcemid was added to induce mitotic arrest. At 72 hours, the cells were harvested using conventional procedures. The cells were then treated with hypotonic 0.07 M KC1 for 12 minutes, fixed, washed with freshly prepared Carnoy's mixture (3:1 [v:v] methanol and acetic acid), and airdried on wet slides. The slides were coded as were the samples and stained with Giemsa solution. A minimum of 50 well-spread metaphases per sample were examined in each sample to determine the number of chromatid breaks (28). Gaps and attenuated regions were disregarded. Mutagen sensitivity was expressed as the average number of breaks per cell (breaks/cell). Higher values of breaks/cell are hypothesized to indicate increased cancer susceptibility.

Statistical analysis

Several statistical approaches were used to assess the quality of the assay results. To assess the possibility of laboratory drift over time, indices of central tendency, individual assay results and cell viability over various dates (thaw date, electrophoresis date, harvest date, culture date), batch number stratified by case and control status were plotted (scatter and box-and-whisker) and visually inspected. Although there was a high degree of heterogeneity in the assay measures from date to date, no clear trend was seen over time that would indicate problematic drift. Coefficients of variation (CVs) were calculated for the eight duplicate and the two sets of four replicate QC samples according to Falk et al (29) for which CVs of 15% or less are considered acceptable. Variation by age at blood collection, time since blood collection, gender, race, smoking status, and the other host characteristics were also assessed in the aggregate and by case-control status.

We used the geometric mean of tail length, tail DNA, CDM and OTM of 100 randomly selected cells per subject as a summary measure to reduce the influence of outliers. No data transformations were used for DRC or breaks/cell outcomes. QQ plots were visually inspected and Kolmogorov-Smirnov tests conducted to assess assumptions of normality.

The association between the assay measures and cancer risk was evaluated by calculating odds ratios (OR) and 95% confidence intervals (CI) based on unconditional logistic regression. All of the assay measures (Comet tail DNA, tail length, CDM, and OTM; DRC; and bleomycin-induced chromatid breaks/cell) were divided into four categories based on the quartiles of the respective distributions in the control group. Other data categorizations including quintiles, tertiles, and dichotomization at the median yielded essentially similar patterns. All models were initially adjusted for the matching variables: age in three categories (55-64, 65-69, 70 years or older), gender, and smoking habits. Of these, age was the only factor to have even a modest impact on the logistic regression point estimates. Other potential confounders including race, education, lung cancer in a first degree relative, history of emphysema, or laboratory variables such as cell viability in culture did not significantly change the point estimates (> 10%), so none of these factors, other than age, were included in the final model. Tests for trend were adjusted for the matching variables and done in two ways: based on the underlying continuous variable and using the quartile-based categorical measure as a score test. All significance tests were two sided and α was set at 0.05. The Statistical Package for the Social Sciences version 16.0 (SPSS, Inc., Chicago, Il) was used for all analyses.

Results

CVs for the eight duplicates and the two sets of four replicate QC samples are shown in Table 1. All the CVs were approximately 15% or less except for the bleomycin assay with a CV of 22% for the two sets of four replicates. The variation in the QC samples was less than the overall variation of the assays in the cases and controls except for the bleomycin assay (data not shown).

Baseline and other characteristics for lung cancer cases and controls are presented in Table 2. The case and control groups did not differ significantly in any of the matching or demographic variables, although cases tended to have a somewhat lower level of education than controls. Calendar time between blood collection and case diagnoses was fairly evenly distributed and 79.5% of cases occurred a year or more after blood donation. The means of all the individual assay measures by case and control status for the demographic variables, calendar time between blood draw and diagnosis, family history of lung cancer, family history of any cancer, history of emphysema, and lung cancer histology did not significantly differ across categories except that among controls, Comet tail DNA tended to increase with age and DRC tended to decrease with increasing age (data not shown).

Lung cancer risks adjusted for age, gender, and smoking history are shown in Table 3. No statistically significant associations with lung cancer were found for the Comet or the DRC assays. However, statistically significant increased lung cancer ORs for the bleomycin assay were observed for increasing quartiles of chromatid breaks/cell relative to the lowest quartile (OR=1.2, 95% confidence interval (CI): 0.5-2.5, OR=1.4, 95% CI: 0.7-3.1, OR=2.1, 95% CI: 1.0-4.4), respectively, $p_{trend} = 0.04$). The association between the bleomycin mutagen sensitivity assay and lung cancer risk was slightly stronger when cases diagnosed within a year of blood collection were excluded ($p_{trend} = 0.02$) and there were no changes in the associations for the other assays when these cases were excluded (data not shown).

Discussion

Our study is the first to prospectively evaluate three widely used mutagen sensitivity assays in relation to lung cancer risk. We showed that increased chromatid breaks/cell in the bleomycin mutagen challenge assay were associated with increased risk of lung cancer. No lung cancer associations were found for the four Comet assays or the DRC assay using BPDE as the test mutagen. Excluding persons diagnosed with lung cancer within one year of blood collection did not change results for the Comet or DRC assays, but sharpened the relationship between the bleomycin assay and lung cancer risk, strengthening the contention that the bleomycin assay reflects some component of cancer predisposition, rather than a state induced in the host by the presence of tumor even at a preclinical stage.

In our study, lung cancer risks rose to about two-fold for those with the greatest numbers of chromatid breaks in the belomycin assay (highest vs. lowest quartile, OR= 2.1). The magnitude of the bleomycin and lung cancer association was, however, less than the generally observed in some previous case-control studies using this assay, where risks up to 10-fold were reported (reviews in: 5-10). In our study, the laboratory variation (CV) was greater for the bleomycin assay than for the Comet and DRC assays which are more mechanized, relying less on reader interpretation. As reader variability introduces a level of error in bleomycin assay scoring, it is possible that the lung cancer risks observed in our study underestimate the true risks, nevertheless, the previous case-control studies were subject to similar reader variation and, thus, the differential in risk between our prospective evaluation and the retrospective studies cannot be entirely attributed to issues of measurement error.

In this study we addressed reverse causation bias by evaluating samples collected before cancer diagnosis, using stored cryopreserved whole blood. As the DNA repair and challenge assays require living cells, the previous lung cancer case-control studies used unfrozen blood sources with direct assay of fresh lymphocytes. It has been difficult to use stored frozen lymphocytes (16) or blood samples, necessary for a prospective evaluation, due to lysed cellular debris and other technical difficulties, despite some reports of success (14). Because our pilot efforts to directly stimulate lymphocytes derived from frozen whole blood were also unsuccessful (AJS, RBH, XW), we developed EBV-transformed LCLs from PLCO cryopreserved whole blood samples and carried out the assays on the cell lines, as an alternative approach. While we reasoned that LCLs from B lymphocytes, despite having undergone immortalization and artificial maintenance in cell culture, retained the genetic endowment of the individual subject (see 25 and references therein), LCLs have some limitations as a suitable material type for the assays we evaluated. For example, some laboratories have reported acceptable and similar reproducibility for peripheral blood lymphocytes and LCLs (30-31), but others have not (32-33). While LCLs currently provide a cost-efficient approach in nested case-control designs for the evaluation of these assays in large-scale prospective studies, we recognize that transformed LCLs may have acquired properties that affect relevance to normal tissues or alter certain assay characteristics.

Our study had several strengths. We used pre-diagnostic samples to avoid reverse-causation bias. The sample identity was blinded to the laboratory investigators and we accounted for age, gender, and smoking status in the study design. The study limitations are a relatively small sample size and, potentially, the use of LCLs as a surrogate material.

In conclusion, we found a modest association of mutagen sensitivity measured by the bleomycin challenge assay and lung cancer risk, indicating that this measure has potential use in lung cancer prediction, particularly if assay variability can be better addressed. Mutagen-sensitivity measured by the Comet and DRC assays was not associated with lung cancer risk in this prospective study.

Acknowledgements

The authors thank Dr. Roni Falk, DCEG, for helpful advice on the coefficient of variation calculations and Drs. Christine Berg and Philip Prorok, Division of Cancer Prevention, National Cancer Institute, the Screening Center investigators and staff of the Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening Trial, Mr. Tom Riley and staff, Information Management Services, Inc., Ms. Barbara O'Brien and staff, Westat, Inc., Ms. Jackie King and staff, BioReliance, Inc. and Tracie Franklin and staff of American Type Culture Collection. This research was supported by the Intramural Research Program of the Division of Cancer Epidemiology and Genetics and by contracts from the Division of Cancer Prevention, National Cancer Institute, NIH, DHHS and in part under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344 and Inter-Agency Agreement-Y1-CP-6010-02. Most importantly, we acknowledge the study participants for their contributions to making this study possible.

References

1. Hsu TC. Genetic instability in the human population: a working hypothesis. Hereditas, 1983;98:1-9.

2. Hsu TC, Johnston DA, Cherry LM, Ramkisson D, Schantz SP, Jessup JM, Winn RJ, Shirley L, Furlong C. Sensitivity to genotyxic effects of bleomycin in humans: Possible relationship to enviromnmental carcinogenesis. Int J Cancer. 1989;43:403-409.

3. Parshad R, Sanford KK, Jones GM. Chromatid damge after G_2 phase X-irradiation of cells from cancer-prone individuals implicates deficiency in DNA repair. Proc Nat Acad Sci USA. 1983;80:5612-5616.

4. Parshad R, Sanford KK, Jones GM. Chromosomal radiosensitivity during G_2 cell-cycle period of skin fibroblasts from individuals with familial cancer. Proc Nat Acad Sci USA. 1985;82:5400-5403.

5. Berwick M, Vineis P. Markers of DNA repair and susceptibility to cancer in humans: an epidemiologic review. J Natl Cancer Inst. 2000;92:874-97.

6. Parshad R, Sanford KK. Radiation-induced chromatid breaks and deficient DNA repair in cancer predisposition. Crit Rev Oncol Hematol. 2001;37:87-96.

7. Spitz MR, Wei Q, Dong Q, Amos CI, Wu X. Genetic susceptibility to lung cancer: the role of DNA damage and repair. Cancer Epidemiol. Biomarkers Prev. 2003;12:689-698.

8. Wu X, Gu J, Sptz MR. Mutagen sensitivity: a genetic predisposition factor for cancer. Cancer Res 2007;67:3493-5.

9. Paz-Elizur T, Sevelya Z, Leitner-Dagan y, Elinger D, Roisman LC, Livneh Z. DNA repair of wxidative DNA damage in human carcinogenesis: potential application for cancer risk assessment and prevention. Cancer Lett 2008;266:60-72

10. Li C, Wang L, Wei Q. DNA repair phenotype and cancer susceptibility—A mini review. Int J Cancer 2009;124:999-1007.

11. Collins A, Harrington V. Repair of oxidative DNA damage: assessing its contribution to cancer prevention. Mutagenesis. 2002;17:489-493.

12. Caporaso N. The molecular epidemiology of oxidative damage to DNA and cancer (editorial). J Natl Cancer Inst. 2003;95:1263-1265

13. Mohrenweiser HW, Wilson DM 3rd, Jones IM. Challenges and complexities in estimated both the functional impact and the disease risk associated with the extensive genetic variation in human DNA repair genes. Mutat Res. 2003;526:93-125.

14. Cheng L, Wang LE, Spitz MR, Wei Q. Cryopreserving whole blood for functional assays using viable lymphocytes in molecular epidemiology studies. Cancer Lett. 2001;166:155-163.

15. Schmezer P, Rajaee-Behbahani N, Risch A, Thiel S, Rittgen W, Drings P, Dienemann H, Kayser KW, Schulz V, Bartsch H. Rapid screening assay for mutagen sensitivity and DNA repair capacity in human peripheral blood lymphocytes. Mutagenesis. 2001;16:25-30.

16. Duthie SJ, Pirie L, Jenkinson AMcE, Narayanan S. Cryopreserved versus freshly isolated lymphocytes in human biomonitoring: endogenous and induced DNA damage, antioxidant status and repair capability. Mutagenesis. 2002;17:211-214.

17. Chao DL, Maley CC, Wu X, Farrow DC, Galipeau PC, Sanchez CA, Paulson TG, Rabinovitch PS, Reid BJ, Spriz MR, Vaughan TL. Mutagen sensitivity and neoplastic progressi in paties with Barrett's esophagus: a prospective analysis. Cancer Epidemiol Biomarkers Prev. 2006;15:1935-40.

18. Bhatti P, Sigurdson AJ, Thomas CB, Iwan A, Alexander BH, Kampa D, Bowen L, Doody MM, Jones IM. No evidence for differences in DNA damage assessed before and after a cancer diagnosis. Cancer Epidemiol Biomarkers Prev. 2008;17:990-4.

19. Orlow I, Park BJ, Mujumdar U, Patel H, Siu-Lau P, Clas BA, Downey R, Flores R, Bains M, Rizk N, Dominguez G, Jani J, Berwick M, Begg CB, Kris MG, Rusch VW. DNA damage and repair capacity in patients with lung cancer: prediction of multiple primary tumors. J Clin Oncol. 2008;26:3560-6.

20. N.P. Singh, M.T. McCoy, R.R. Tice, E.L. Schneider, A simple technique for quantitation of low levels of DNA damage in individual cells, Exp. Cell Res. 175 (1988) 184-191.

21. Athas AF, Dedayati M, Matanoski GM, Farmer ER, Grossman L. Development and field-test validation of an assay for DNA repair in circulating human lymphocytes. Cancer Res. 1991;51:5786-5793;

22. Gohagen JK, Prorok PC, Hayes RB, Kramer BS, for the PLCO Project Team; The Prostate, Lung, Colorectal and Ovarian (PLCO) cancer screening trial of the National Cancer Institute: history, organization , and status. Controlled Clinical Trials 2000;21:251S-272S.

23. Hayes RB, Smith CO, Huang WY, Read Y, Kopp WC. Whole blood cryopreservation in epidemiological studies. Cancer Epidemiol Biomarkers Prev. 2002;11:1496-1498.

24. Hayes RB, Sigurdson A, Moore L, Peters U, Huang W-Y, Pinsky P, Reding d, Gelmann EP, Rothman N, Pfeiffer RM, Hoover RN, Berg CD. Methods for etiologic and early marker investigations in the PLCO trial. Mutat Res 2005;592:147-154.

25. Sigurdson AJ, Hauptmann M, Alexander BH, Doody MM, Thomas CB, Struewing JP, Jones IM. DNA damage among thyroid cancer and multiple cancer cases, controls, and long-lived individuals. Mutat Res (Genetic Toxicology and Environmental Mutagenesis) 2005;586:173-188.

26. Wei Q, Spitz MR, Gu J, Cheng L, Xu X, Strom SS, Kripke ML, Hsu TC. DNA repair capacity correlates with mutagen sensitivity in lymphoblastoid cell lines. Cancer Epidemiol Biomarkers Prev. 1996;5:199-204.

27. Wei Q, Cheng L, Amos CI, Wang LE, Guo Z, Hong WK, Spitz MR Repair of tobacco carcinogen-induced DNA adducts and lung cancer risk: a molecular epidemiologic study. J Natl Cancer Inst. 2000;92:1764-72.

28. Lee JJ, Trizna Z, Hsu TC, Spitz MR, Hong WK. A statistical analysis of the reliability and classification error in application of the mutagen sensitivity assay. Cancer Epidemiol Biomarkers Prev. 1996;5:191-7.

29. Falk RT, Gail MH, Fears TR, et al., Reproducibility and validity of radioimmunoassay for urinary hormones and metabolites in pre-and postmenopausal women, Cancer Epidemiol Biomarkers Prev. 1999;8:567-577.

30. Cloos J, Temmink O, Ceelen M, Snel MHJ, Leemans CR, Braakhuis BJM. Involvement of cell cycle control in bleomycin-induced mutagen sensitivity. Environ Mol Mutagenesis 2002;40:79-84.

31. Hsu TC, Shillitoe EJ, Cherry LM, Lin Q, Schantz SP, Furlong C. Cytogenetic characterization of 20 lymphoblastoid cell lines derived from human individuals differing in bleomycin sensitivity. *In Vitro* Cell Dev Biol 1990;26:80-84.

32. Baeyens A, Thierens H, Vandenbulcke K, De Ridder L, Vral A. The use of EBV-transformed cell lines of breast cancer patients to measure chromosomal radiosensitivity. Mutagenesis 2004;19:285-290.

33. Zijno A, Porcedda P, Saini F, Allione A, Garofalo B, Marcon F, Guarrera S, Turinetto V, Minieri V, Funaro A, Crebelli R, Giachino C, Matullo G. Unsuitability of lymphoblastoid cell lines as surrogate of cryopreserved isolated lymphocytes for the analysis of DNA double-strand break repair activity. Mutat Res. 2010;684:98-105.

Table I. Coefficients of variation for blinded quality controls samples included in shipments to each laboratory

Assay Name	Eight Duplicates	Two sets of four replicates
% DNA in Comet tail	8.0 %	11.8 %
Comet tail length	7.1 %	13.7 %
Comet distributed moment (CDM)	5.2 %	6.8 %
Olive tail moment (OTM)	8.4 %	15.4 %
DNA repair capacity (DRC)	5.2 %	8.4 %
Bleomycin sensitivity (breaks/cell)	15.1 %	22.6 %

N.B. The alkaline Comet Assay was performed on unchallenged cells and measured endogenous levels of DNA damage. The DNA repair capacity measure used the host-cell reactivation assay with the mutagen benzo(a)pyrene diol epoxide. The mutagen sensitivity assay used the mutagen bleomycin and measured the number of chromatid breaks per cell.

Table II. Baseline characteristics of lung cancer cases and controls nested within the Prostate, Lung, Colorectal and Ovarian Cancer Screening Trial

	Controls (n=117)		Cases (n=117)		
Characteristic at the time of blood collection	N	%	N	%	p-value
Age in years*					
55-64	30	25.6	30	25.6	1.00
65-69	37	31.6	37	31.6	
70-79	50	42.7	50	42.7	
Gender*					
Male	81	69.2	81	69.2	1.00
Female	36	30.8	36	30.8	
Cigarette smoking status*					
Never smoked cigarettes[†]	7	6.0	7	6.0	1.00
Quit 10+ years ago and cigarettes/day ≤ 1 pack	8	6.8	8	6.8	
Quit 10+ years ago and cigarettes/day > pack	27	23.1	27	23.1	
Current or quit < 10 years ago, and cigarettes/day ≤ 1 pack	39	33.3	39	33.3	
Current or quit < 10 years ago, and cigarettes/day > 1 pack	36	30.8	36	30.8	
Race					
White, non-Hispanic	112	95.7	109	93.2	
Nonwhite or Hispanic	5	4.3	8	6.8	0.39
Education					
11 years or less	12	10.3	13	11.1	0.70
12 years or completed high school	23	19.7	28	23.9	
Post-high school other than college, or some college	41	35.0	43	36.8	
College graduate or postgraduate	41	35.0	33	28.2	
Lung cancer reported in first-degree relatives[‡]					
No	97	84.3	98	84.5	0.64
Yes	15	13.0	13	11.2	
Unknown	3	2.6	5	4.3	
History of emphysema[‡]					
No	104	92.0	96	88.9	0.43
Yes	9	8.0	12	11.1	
Months between blood collection and lung cancer diagnosis					NA
3-11	NA		24	20.5	
12-23	NA		28	23.9	
24-35	NA		26	22.2	
36-47	NA		24	20.5	
48+	NA		15	12.8	
Lung cancer histology					
Adenocarcinoma	NA		39	33.3	NA
Squamous	NA		27	23.1	
Small cell	NA		21	17.9	
Non-small cell, not otherwise specified	NA		8	6.8	
Large cell	NA		7	6.0	
Other[§]	NA		15	12.8	

NB: NA is not applicable
* Matching variables
† Two cases and one control reported having smoked cigars but not cigarettes
‡ May not sum to 117 cases or 117 controls due to missing data
§ Four cases bronchioalveolar adenocarcinoma, two cases acinar adenocarcinoma, two cases carcinoma not otherwise specified, one case intermediate cell carcinoma, one case adenosquamous carcinoma, five cases not available.

Table III. Adjusted odds ratios and 95% confidence intervals for assay measures and lung cancer risk in a nested case-control study within the Prostate, Lung, Colorectal, and Ovarian cancer screening trial

Assay type by quartile	*Number of controls*	*Number of cases*[*]	*Odds Ratio*[†]	*95% Confidence Interval*		*P for trend*[‡] *Score*	*Continuous*
Comet Tail DNA (%)							
(3.6 - 6.1)	29	21	1.00	Referent			
(6.2 - 7.1)	30	32	1.49	0.69	3.21		
(7.2 - 8.4)	29	36	1.71	0.81	3.63		
(8.5 - 17.4)	29	25	1.20	0.54	2.65	0.60	0.92
Comet Tail Length (μm)							
(15.0 - 29.4)	29	27	1.00	Referent			
(29.5 - 33.2)	29	28	1.01	0.47	2.18		
(33.3 - 36.0)	30	25	0.90	0.42	1.91		
(36.1 - 45.0)	29	34	1.26	0.60	2.63	0.61	0.75
Comet Distributed Moment							
(15.1 - 17.4)	29	31	1.00	Referent			
(17.5 - 18.3)	30	29	0.90	0.43	1.89		
(18.4 - 19.5)	29	28	0.90	0.43	1.88		
(19.6 - 27.8)	29	26	0.85	0.40	1.77	0.67	0.80
Olive Tail Moment							
(0.8 - 1.2)	29	23	1.00	Referent			
(1.3 - 1.5)	30	36	1.57	0.74	3.30		
(1.6 - 1.7)	29	24	1.04	0.48	2.26		
(1.8 - 4.5)	29	31	1.39	0.65	2.97	0.68	0.92
DNA Repair Capacity (%)[§]							
(12.5 - 20.2)	29	28	1.00	Referent			
(10.6 - 12.4)	28	31	1.15	0.55	2.41		
(9.0 - 10.5)	31	31	1.03	0.50	2.16		
(5.0 - 8.9)	29	27	0.96	0.45	2.04	0.86	0.67
Bleomycin Mutagen Sensitivity (breaks/cell)							
(0.08 - 0.31)	31	22	1.00	Referent			
(0.32 - 0.45)	32	26	1.15	0.54	2.45		
(0.46 - 0.57)	26	26	1.41	0.65	3.08		
(0.58 - 1.26)	28	41	2.09	1.00	4.37	0.04	0.05

N.B. Assay measures were divided into quartiles based on the control distribution. The alkaline Comet Assay was performed on unchallenged cells and measured endogenous levels of DNA damage. The Comet measures of Comet Tail Moment and Olive Tail Moment do not have units. The DNA repair capacity measure used the host-cell reactivation assay with the mutagen benzo(a)pyrene diol epoxide and is the percent of repaired plasmids relative to undamaged plasmids. The mutagen sensitivity assay used the mutagen bleomycin and measured the number of chromatid breaks per cell.

[*] The number of cases may not sum to total due to poor growth of a few lymphoblastoid cell lines in some laboratories.

[†] Adjusted for the matching variables age, gender, and smoking history.

[‡] p for trends are a 1 df score test and based on the continuous underlying variable

[§] Quartile sequence is reversed because increased DNA repair capacity relative to lower repair is considered the referent group